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Supporting Information

A Water-Bridged H-Bonding Network Contributes to the Catalysis of the SAM-Dependent C-Methyltransferase HcgC

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Supporting Information

Materials and methods

Materials

S-(5'-adenosyl)-L-methionine (SAM) chloride dihydrochloride and *S*-(5'-adenosyl)-L-homocysteine (SAH) were purchased from Sigma-Aldrich. Sodium chloride, potassium chloride, potassium hydroxide, magnesium chloride, hydrogen chloride, Tris (hydroxymethyl) aminomethane, methanol, ethanol, kanamycin, 3-(*N*-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)piperazine-1- ethanesulfonic acid (HEPES) were obtained from Roth. Isopropyl β-D-thiogalactopyranoside (IPTG) was from Thermo. Pyridinol substrate **2** was chemically synthesized.[1]

Construction of the expression vector for HcgC production

The *hcgC* gene from *Methanococcus maripaludis* strain S2 (MMP1498, GenBank accession number NP_988618.1) was used for heterologous production of HcgC in this work. The *hcgC* gene was designed and synthesized by Genscript to optimize the codon usage and mRNA structure for expression in *Escherichia coli* and to have C-terminal extension with His-tag. The synthesized gene was inserted between the *Nde*I and *Sal*I sites of pET-24b(+). The *hcgC* sequences are shown below. The start codon is marked with yellow background. The *Nde*I and *Sal*I recognition sites are indicated with underline and double-underline, respectively. The codon for the mutation in the enzyme variants is indicated with light-blue background.

The wild type HcgC:

CATATGAACTACGGCATTACCGAAAGCGTGAAAACGACCCGCAGCAAAATCAAA
ATCAAAGATATTGTGTCCGATGTGGTGGAAAAGAAAGCGAACGCCATCAAATAT
TTTCTGGAAGGCGAAGAATTTAAACAGGCAATTGTGTTTGGCGCTTACCTGTCAG

GTTCGTATATCGCGTACTCACTGCTGAAAGATTGCGAAGAAGTCATTATCGTGGA

CATTCAGCCGCATCTGAAAGATATTCTGTTCAACGACGGTATCAAATTCATGGAT

CTGAACAAACTGCAACTGGAACTGCGTAACGGCACCAGCATCAATCCGGATCTG

GTGATTGACCTGACGGGTATCGGCGGTGTTAGTCCGGATCTGATTTCCAAATTCA

ATCCGAAAGTTCTGATCGTCGAAGATCCGAAAGGCAACCACGACAAAGGTATCT

CTAAAATCGATAACACCGACAAACGTCTGTGCGTGGGCGCGAAAAAAGGTGTTC

TGAAAACCTATCGCAGCTCTAAATTTAGCAAAACGTCTGGCACCATGACCCTGGT

GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG

TATACCATCCCGAATCTGAAATACTTTGAGGGTACGGTCTTCCATGAGAAAAACG

TGAAAAAATTCCTGACCGAACTGAATATGTCCGCCATTACCGTTAGTTCCATCGA

TCACGTCGAATACGAACTGGAAGAAATCCTGTCAAAAAACATCAGCCGTGTGGA

CTCGTTCGTGAAAGAATTTGTCGAC

The T6V mutant of HcgC:

CATATGAACTACGGCATTGTGGAAAGCGTGAAAACGACCCGCAGCAAAATCAAA

ATCAAAGATATTGTGTCCGATGTGGTGGAAAAGAAAGCGAACGCCATCAAATAT

TTTCTGGAAGGCGAAGAATTTAAACAGGCAATTGTGTTTGGCGCTTACCTGTCAG

GTTCGTATATCGCGTACTCACTGCTGAAAGATTGCGAAGAAGTCATTATCGTGGA

CATTCAGCCGCATCTGAAAGATATTCTGTTCAACGACGGTATCAAATTCATGGAT

CTGAACAAACTGCAACTGGAACTGCGTAACGGCACCAGCATCAATCCGGATCTG

GTGATTGACCTGACGGGTATCGGCGGTGTTAGTCCGGATCTGATTTCCAAATTCA

ATCCGAAAGTTCTGATCGTCGAAGATCCGAAAGGCAACCACGACAAAGGTATCT

CTAAAATCGATAACACCGACAAACGTCTGTGCGTGGGCGCGAAAAAAGGTGTTC

TGAAAACCTATCGCAGCTCTAAATTTAGCAAAACGTCTGGCACCATGACCCTGGT

GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG

TATACCATCCCGAATCTGAAATACTTTGAGGGTACGGTCTTCCATGAGAAAAACG

TGAAAAAATTCCTGACCGAACTGAATATGTCCGCCATTACCGTTAGTTCCATCGA

TCACGTCGAATACGAACTGGAAGAAATCCTGTCAAAAAACATCAGCCGTGTGGA

CTCGTTCGTGAAAGAATTTGTCGAC

The Y51F mutant of HcgC:

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TTTCTGGAAGGCGAAGAATTTAAACAGGCAATTGTGTTTGGCGCTTTTCTGTCAG

GTTCGTATATCGCGTACTCACTGCTGAAAGATTGCGAAGAAGTCATTATCGTGGA

CATTCAGCCGCATCTGAAAGATATTCTGTTCAACGACGGTATCAAATTCATGGAT

CTGAACAAACTGCAACTGGAACTGCGTAACGGCACCAGCATCAATCCGGATCTG

GTGATTGACCTGACGGGTATCGGCGGTGTTAGTCCGGATCTGATTTCCAAATTCA

ATCCGAAAGTTCTGATCGTCGAAGATCCGAAAGGCAACCACGACAAAGGTATCT

CTAAAATCGATAACACCGACAAACGTCTGTGCGTGGGCGCGAAAAAAGGTGTTC

TGAAAACCTATCGCAGCTCTAAATTTAGCAAAACGTCTGGCACCATGACCCTGGT

GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG

TATACCATCCCGAATCTGAAATACTTTGAGGGTACGGTCTTCCATGAGAAAAACG

TGAAAAAATTCCTGACCGAACTGAATATGTCCGCCATTACCGTTAGTTCCATCGA

TCACGTCGAATACGAACTGGAAGAAATCCTGTCAAAAAACATCAGCCGTGTGGA

CTCGTTCGTGAAAGAATTTGTCGAC

The S175A mutant of HcgC:

CATATGAACTACGGCATTACCGAAAGCGTGAAAACGACCCGCAGCAAAATCAAA

ATCAAAGATATTGTGTCCGATGTGGTGGAAAAGAAAGCGAACGCCATCAAATAT

TTTCTGGAAGGCGAAGAATTTAAACAGGCAATTGTGTTTGGCGCTTACCTGTCAG

GTTCGTATATCGCGTACTCACTGCTGAAAGATTGCGAAGAAGTCATTATCGTGGA

CATTCAGCCGCATCTGAAAGATATTCTGTTCAACGACGGTATCAAATTCATGGAT

CTGAACAAACTGCAACTGGAACTGCGTAACGGCACCAGCATCAATCCGGATCTG

GTGATTGACCTGACGGGTATCGGCGGTGTTAGTCCGGATCTGATTTCCAAATTCA

ATCCGAAAGTTCTGATCGTCGAAGATCCGAAAGGCAACCACGACAAAGGTATCT

CTAAAATCGATAACACCGACAAACGTCTGTGCGTGGGCGCGAAAAAAGGTGTTC

TGAAAACCTATCGCAGCTCTAAATTTAGCAAAACGGCGGGCACCATGACCCTGGT

GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG

TATACCATCCCGAATCTGAAATACTTTGAGGGTACGGTCTTCCATGAGAAAAACG

TGAAAAAATTCCTGACCGAACTGAATATGTCCGCCATTACCGTTAGTTCCATCGA

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CTCGTTCGTGAAAGAATTTGTCGAC

The T179V mutant of HcgC:

CATATGAACTACGGCATTACCGAAAGCGTGAAAACGACCCGCAGCAAAATCAAA

ATCAAAGATATTGTGTCCGATGTGGTGGAAAAGAAAGCGAACGCCATCAAATAT

TTTCTGGAAGGCGAAGAATTTAAACAGGCAATTGTGTTTGGCGCTTACCTGTCAG

GTTCGTATATCGCGTACTCACTGCTGAAAGATTGCGAAGAAGTCATTATCGTGGA

CATTCAGCCGCATCTGAAAGATATTCTGTTCAACGACGGTATCAAATTCATGGAT

CTGAACAAACTGCAACTGGAACTGCGTAACGGCACCAGCATCAATCCGGATCTG

GTGATTGACCTGACGGGTATCGGCGGTGTTAGTCCGGATCTGATTTCCAAATTCA

ATCCGAAAGTTCTGATCGTCGAAGATCCGAAAGGCAACCACGACAAAGGTATCT

CTAAAATCGATAACACCGACAAACGTCTGTGCGTGGGCGCGAAAAAAGGTGTTC

TGAAAACCTATCGCAGCTCTAAATTTAGCAAAACGTCTGGCACCATGGTCCTGGT

GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG

TATACCATCCCGAATCTGAAATACTTTGAGGGTACGGTCTTCCATGAGAAAAACG

TGAAAAAATTCCTGACCGAACTGAATATGTCCGCCATTACCGTTAGTTCCATCGA

TCACGTCGAATACGAACTGGAAGAAATCCTGTCAAAAAACATCAGCCGTGTGGA

CTCGTTCGTGAAAGAATTTGTCGAC

The E209Q mutant of HcgC:

CATATGAACTACGGCATTACCGAAAGCGTGAAAACGACCCGCAGCAAAATCAAA

ATCAAAGATATTGTGTCCGATGTGGTGGAAAAGAAAGCGAACGCCATCAAATAT

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ATCCGAAAGTTCTGATCGTCGAAGATCCGAAAGGCAACCACGACAAAGGTATCT

CTAAAATCGATAACACCGACAAACGTCTGTGCGTGGGCGCGAAAAAAGGTGTTC

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GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG

TATACCATCCCGAATCTGAAATACTTTCAGGGTACGGTCTTCCATGAGAAAAACG

TGAAAAAATTCCTGACCGAACTGAATATGTCCGCCATTACCGTTAGTTCCATCGA

TCACGTCGAATACGAACTGGAAGAAATCCTGTCAAAAAACATCAGCCGTGTGGA

CTCGTTCGTGAAAGAATTTGTCGAC

The S233A mutant of HcgC:

CATATGAACTACGGCATTACCGAAAGCGTGAAAACGACCCGCAGCAAAATCAAA
ATCAAAGATATTGTGTCCGATGTGGTGGAAAAGAAAGCGAACGCCATCAAATAT
TTTCTGGAAGGCGAAGAATTTAAACAGGCAATTGTGTTTGGCGCTTACCTGTCAG
GTTCGTATATCGCGTACTCACTGCTGAAAGATTGCGAAGAAGTCATTATCGTGGA
CATTCAGCCGCATCTGAAAGATATTCTGTTCAACGACGGTATCAAATTCATGGAT
CTGAACAAACTGCAACTGGAACTGCGTAACGGCACCAGCATCAATCCGGATCTG
GTGATTGACCTGACGGGTATCGGCGGTGTTAGTCCGGATCTGATTTCCAAATTCA
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GGTGGATATTATCATGGACTCATGTCGCGAAATTAACGAACTGGATTCGGTTCTG
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TCACGTCGAATACGAACTGGAAGAAATCCTGTCAAAAAACATCAGCCGTGTGGA
CTCGTTCGTGAAAGAATTTGTCGAC

Expression and purification

The HcgC expression vector pET24b(+) harboring the *hcgC* gene and its mutated genes were transferred into *E.coli* BL 21Star (DE3) strain (Invitrogen) and cultivated in LB medium at 37 ºC with 50 µg/mL kanamycin. An over-night grown pre-culture (50 ml) was inoculated into 2-liter LB medium in 5-liter Erlenmeyer flask, which was stirred (500 rpm) using a stirrer bar. When OD_{600} of the culture reached to 0.8-1.0, 1-mM IPTG (final concentration) was added to the medium to induce the production of the HcgC protein. After 4-6 hours cultivation at 37 °C, the *E. coli* cells were harvested by centrifugation at 15,000 ×g for 40 min at 4 °C. The harvested cells were stored at −75 ºC before use. Protein purification was performed under the

aerobic condition. The frozen cell was suspended in the 45-mL of 50 mM Tris/HCl pH 7.0, 20 mM imidazole, 250 mM KCl (buffer A) and disrupted on ice by sonication (Bandelin, 200W) using KE76 tip with 70% power with 50% cycle for 1 min (10 times with 1 min intervals). The crude cell extract was centrifuged at 18,000 ×g for 40 min at 4 °C. The supernatant was loaded onto a HisTrap HP column, which is packed with Ni Sepharose High Performance, equilibrated with buffer A. Target protein HcgC was eluted with linear gradient (0-100%) of 50 mM Tris/HCl pH 7.0 containing 250-mM NaCl and 500-mM imidazole (buffer B). The collected fractions were concentrated using ultrafilter (10 kDa cut-off filter, Millipore) to a final concentration of around 30 mg/mL and then stored at −75 ºC. Protein concentration was determined by the Bradford method using InstantBlue staining-solution from Expedeon. The mutated proteins were purified with the same method as the wild-type HcgC protein.

Enzyme activity assay

The HcgC enzyme-reaction solution contained 10 mM MOPS/KOH pH 7.0, 1.0 mM SAM, variable concentrations of pyridinol **2** (0−0.2 mM) and 1.0 μM HcgC or 1-10 μM mutated enzyme. The enzyme reaction was performed aerobically under air at 37 ºC. The reaction was started by addition of the enzyme solution. Product formed was determined using HPLC (JASCO) equipped with the Polar RP 80Å column (Phenomenex). The reaction solution (100 μl) was filtrated using 0.2-μm pore-size filter and 20 μl was loaded to the column equilibrated with H_2O (pH adjusted to 4.0 with HCl). Then, the column was washed with 2.5 ml H_2O (pH 4.0) and then eluted with 0-100% methanol gradient in a 12.5 ml elution volume. The flow rate was 0.5 ml/min. The substrate and product were eluted at around 76% and 80% methanol, respectively. As the substrate **2** and product **3** have almost the same absorbance at 288 nm, the conversion rate from substrate to product was calculated from the ratio of [area of **3**] / [total area of **2** and **3**].[1]

Crystallization

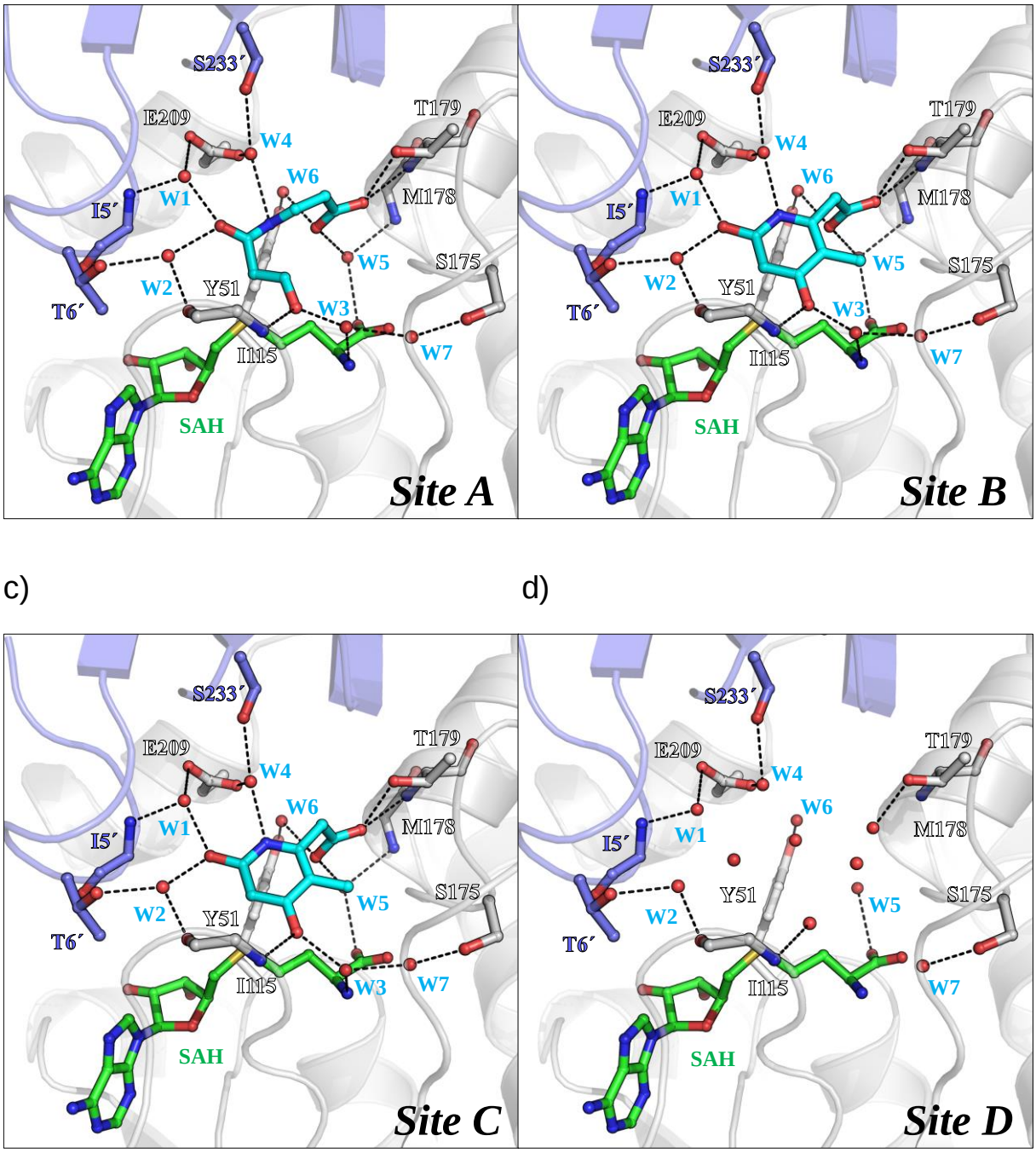
Crystallization of HcgC were performed under air. The buffer of HcgC was exchanged with 10 mM MOPS/KOH pH 7.0 by concentration-dilution cycles (three times) of the protein solution using 10-kDa cut filter and finally concentrated to ~5 mg/ml. The 96/24-well crystal plate, Combiclover Junior (Jena Bioscience), was used for crystallization. The drop consists of 1 µl of enzyme solution containing ~5 mg/ml HcgC, 2 mM pyridinol **2** and 2 mM SAM or SAH and 1 µl of the reservoir solution. The first hits were obtained in a reservoir solution containing 100 mM Tris/HCl pH 8.5, 40% polyethylene glycol (PEG) 400 and 200 mM lithium sulfate (Li_2SO_4), and 100 mM HEPES/NaOH pH 7.5, 0.2 M NaCl, and 35% MPD (2-methyl-2,4-pentanediol) within several weeks. Co-crystals of HcgC with SAM and pyridinol came from a crystallization solution containing 50% v/v PEG 400, 100 mM Na acetate pH 4.5, 200 mM Li_2SO_4. HcgC cocrystallized with SAH and pyridinol came from a crystallization solution containing 40% v/v PEG 400, 100 mM Tris/HCl pH 8.5 and 200 mM Li_2SO_4. The freshly fished crystals (growth after 2 days) were obtained from the drops containing 2-mM SAH and 2-mM pyridinol in 100 mM HEPES/NaOH pH 7.0, 0.1 M NaCl, and 30% MPD. Crystals of the HcgC apoenzyme were obtained from a solution containing 100 mM HEPES/NaOH pH 7.5, 0.1 M NaCl, and 30% MPD. The apoenzyme crystals were soaked overnight in the crystallization solution, which contained 2 mM SAH and 3 mM pyridinol **2**.

Structure analysis

The crystals were cryo-protected by soaking with 30 % glycerol (v/v) in the crystallization solution for 3-5 seconds. The diffraction experiments were performed at 100 K on beamline X10SA equipped with a PILATUS 6M detector at the Swiss Light Source (Villigen). The data were processed with XDS[2] and scaled with SCALA from the ccp4 suite.[3] The structure was

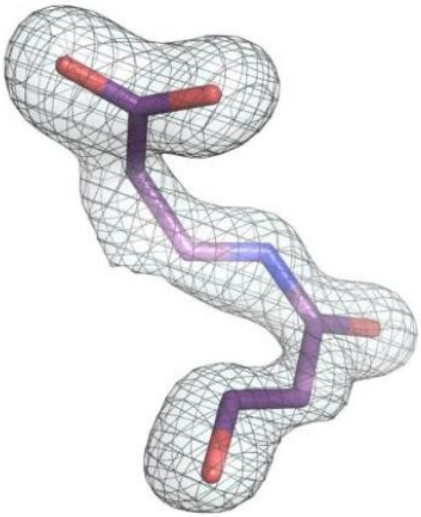
determined with PHASER[4] using the HcgC model from *Methanocaldococcus jannaschii* in complex with SAM (PDB code 2JJF) as a template. The molecular replacement calculation succeeded by using separately the N and C-terminal domain instead of the complete polypeptide chain. The model was manually constructed with COOT[5] and refined by PHENIX.[4] The final model was validated by using the MolProbity server (http://molprobity.biochem.duke.edu).[6] Data collection and refinement statistics of the model are listed in Table S2. The figures were generated and rendered with PyMOL (Version 1.5, Schrödinger, LLC).

a) b)



c) d)

Figure S1. Active sites of the four monomers in the HcgC homotetramer. (a) SAH and a linear compound, (b) SAH and **2,** (c) SAH and a mixture of **2** and a linear compound and (d) SAH.



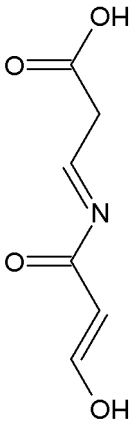


Figure S2. The linear compound found in one of the active site of the tetrameric HcgC. The chemical structure was estimated from the structure of **2** and the interactions with amino acids. The $2F_o$-F_c map is contoured at 1.5 σ.

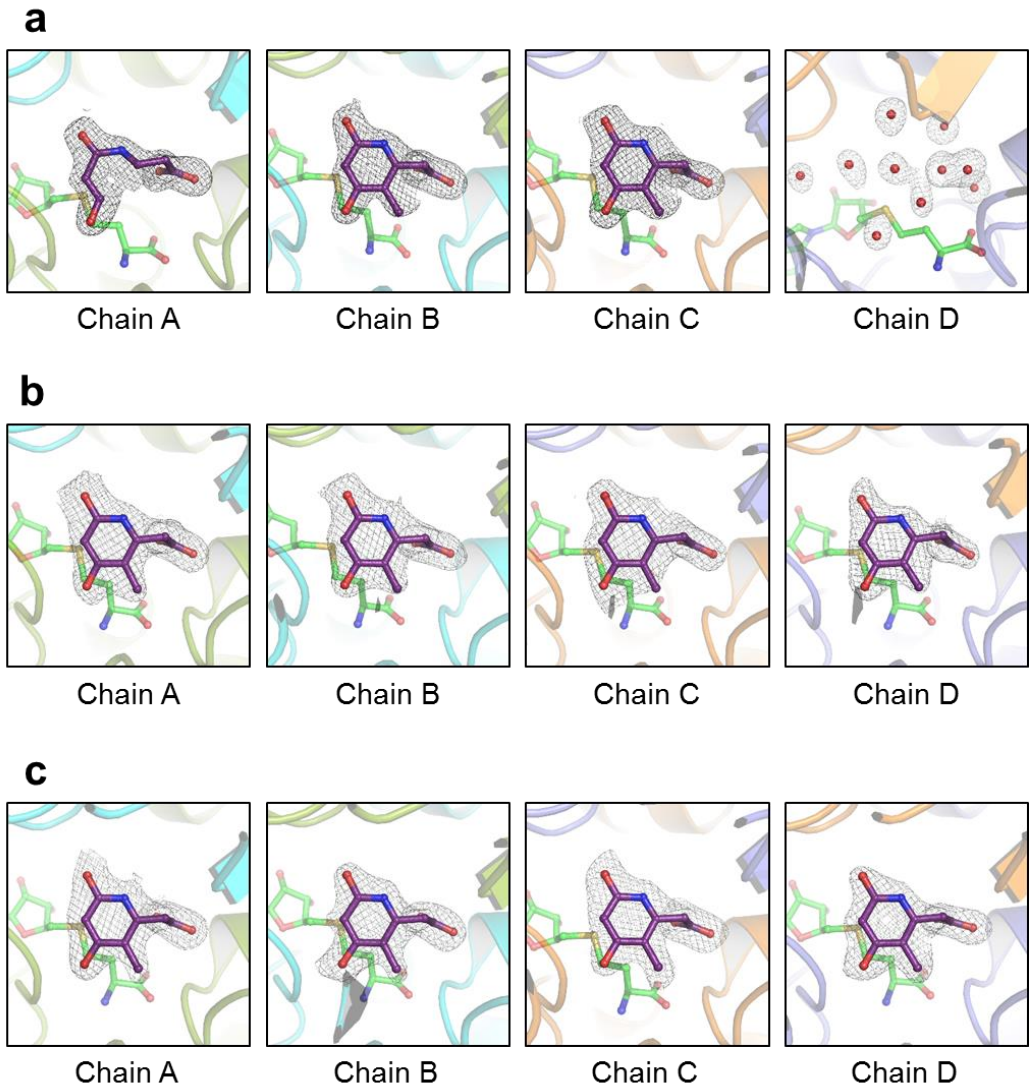
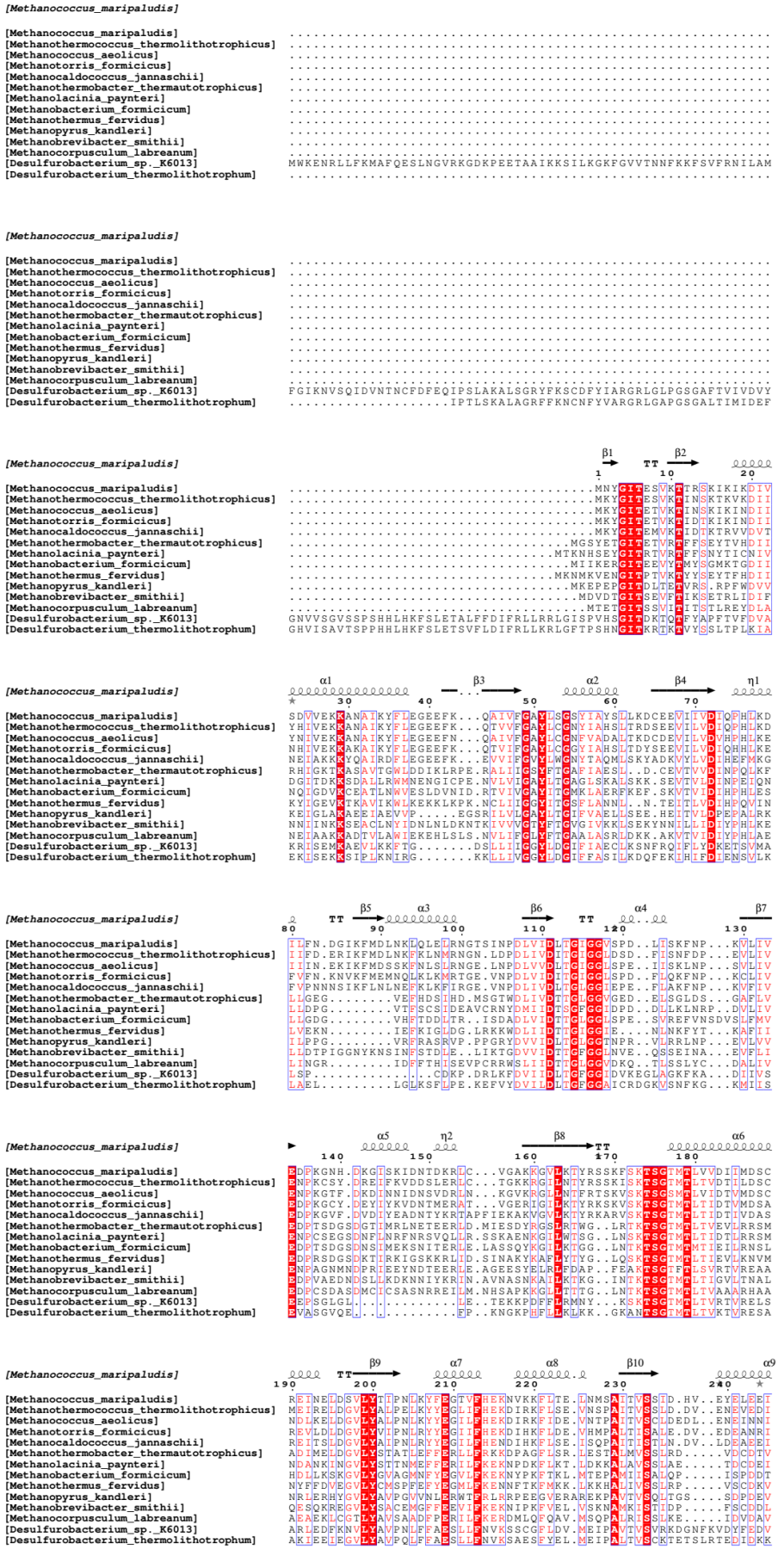


Figure S3. Active site structure of HcgC from *M. maripaludis* in complex with SAH and pyridinol **2**. The four active sites of the dimer of homodimer (chain A-D) are shown in the panels. (a) Structure from ``old´´ crystals containing SAH and pyridinol in solution (1.7 Å resolution). (b) Structure of the crystal freshly fished after two days within less than two days (2.0 Å resolution). (c) Structure of the HcgC-apoenzyme crystal soaked with the substrates (2.05 Å resolution).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[Methanothermobacter_thermautotrophicus]
[Methanolacinia_paynteri]
[Methanobacterium_formicicum]
[Methanothermus_fervidus]
[Methanopyrus_kandleri]
[Methanobrevibacter_smithii]
[Methanocorpusculum_labreanum]
[Desulfurobacterium_sp._K6013]
[Desulfurobacterium_thermolithotrophum]

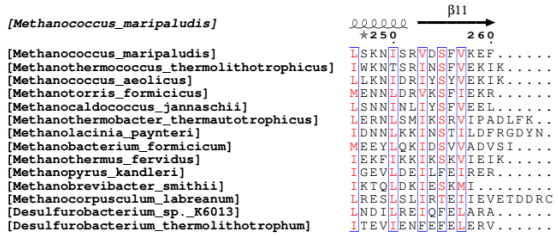

Figure S4. Comparison of primary structures of HcgC.

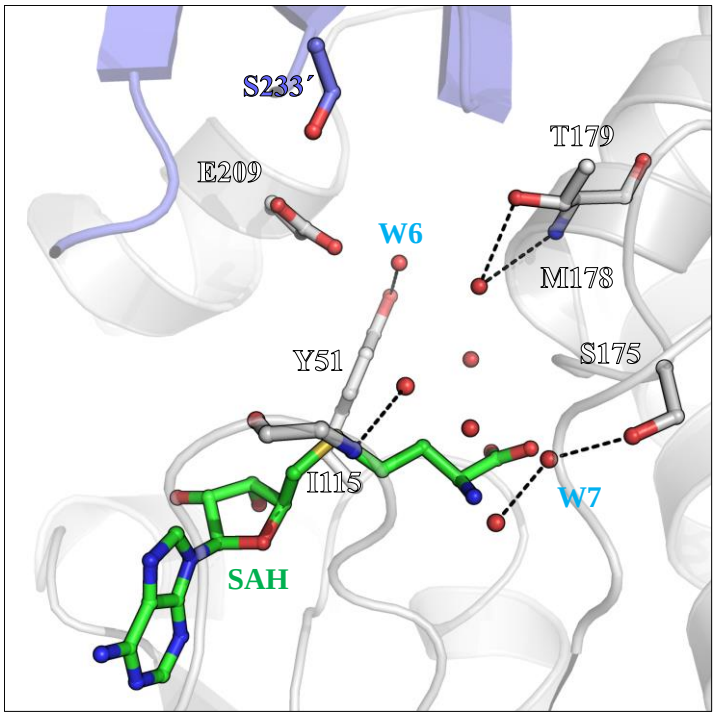

Figure S5. The active-site structure of HcgC crystallized in the presence of SAM and **2**. In the crystallization drop, SAM and substrate **2** converted to SAH and the methylated product **3** by HcgC. Accordingly, HcgC is active under the crystallization conditions.

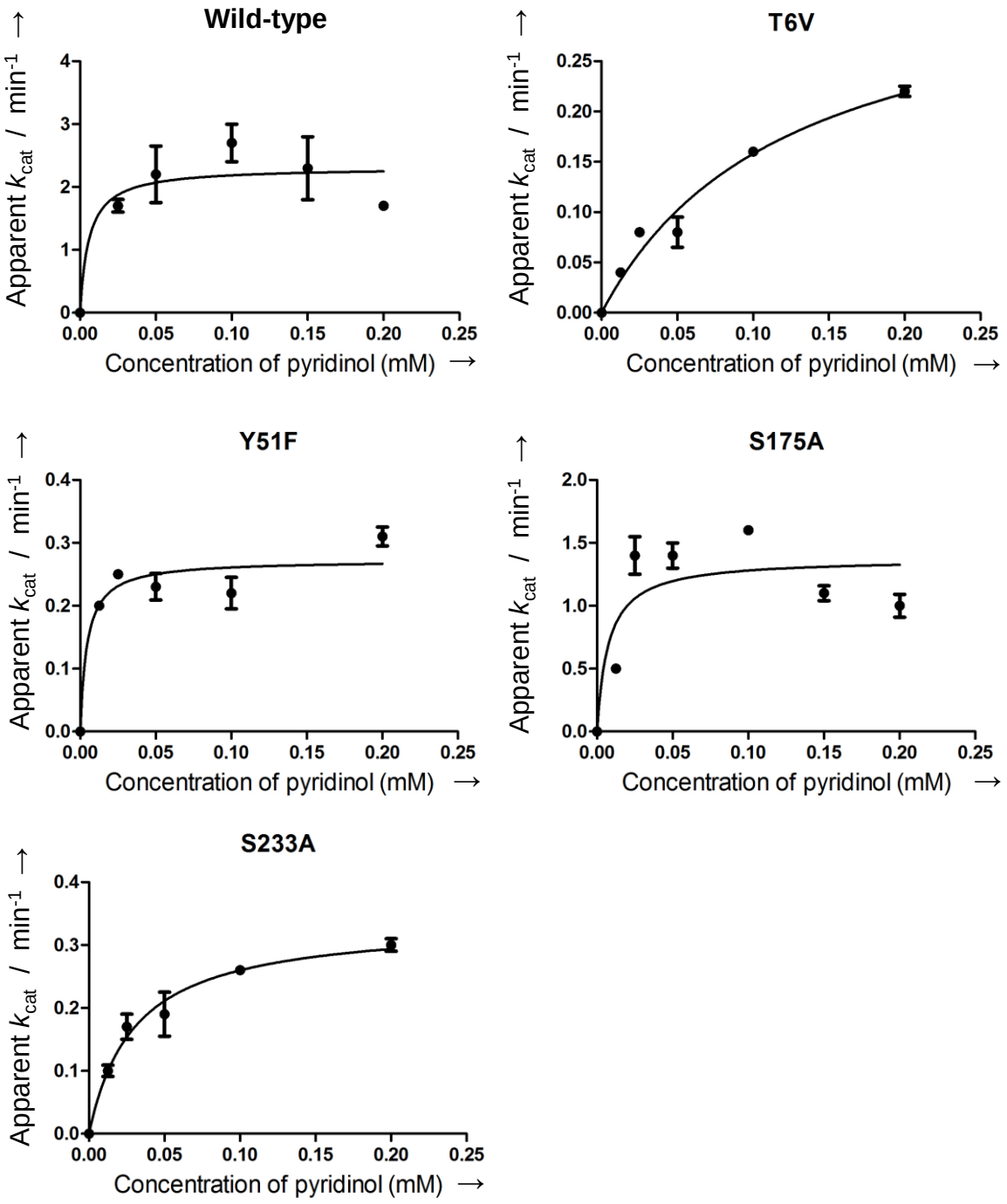

Figure S6. Kinetic data of the wild-type and mutated enzymes. The standard error of at least three measurements was calculated with the Standard Error Calculator. The assay mixture contained 1 mM SAM and variable concentrations of substrate **2**.

Table 1. Statistics of the crystal structure analysis.

	HcgC co-crystallized with SAM+pyridinol	HcgC co-crystallized with SAH+pyridinol	HcgC freshly co-crystallized with SAH+pyridinol	HcgC apo soaked with SAH+pyridinol
Data collection				
Wavelength (Å)	0.99999	0.99999	1.00001	1.00001
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Resolution (Å)	50 – 1.75 (1.84 – 1.75)	50 – 1.70 (1.79 – 1.70)	50 – 2.10 (2.21 – 2.10)	50 – 2.05 (2.16 – 2.05)
Cell dimensions				
a, b, c (Å)	73.2, 77.7, 101.0	72.7, 83.0, 96.1	71.9, 83.5, 100.0	72.6, 81.0, 97.8
α, β, γ (°)	90.0, 110.9, 90.0	90.0, 108.0, 90.0	90.0, 109.5, 90.0	90.0, 108.2, 90.0
R_{merge} (%)[a]	8.2 (64.6)	7.1 (56.2)	5.4 (54.4)	10.3 (71.7)
R_{pim} (%)[a]	4.1 (33.1)	4.3 (33.4)	3.9 (41.2)	7.2 (49.8)
$CC_{1/2}$[a]	99.7 (42.1)	99.7 (58.9)	99.8 (80.4)	99.3 (60.0)
I/σ_I[a]	9.7 (2.2)	9.5 (2.1)	11.5 (1.7)	6.2 (1.4)
Completeness (%)[a]	99.7 (100.0)	99.6 (99.9)	97.9 (98.8)	96.7 (98.0)
Redundancy[a]	4.8 (4.7)	3.7 (3.8)	2.6 (2.5)	2.9 (2.9)
Number of unique reflections[a]	106203 (15490)	119006 (17367)	63739 (9372)	65248 (9601)
Refinement				
Resolution (Å)	50.0 – 1.75	50.0 – 1.70	50.0 – 2.10	30.0 – 2.05
Number of reflections	106177	118942	63705	65131
R_{work}/R_{free}[b] (%)	16.7 / 19.3	15.9 / 19.2	20.2 / 23.3	19.7 / 23.6
Number of atoms				
Protein	8280	8359	8302	8263
Ligands/ions	200	205	208	212
Solvent	699	839	522	470
Mean B-value ($Å^2$)	38.0	36.9	56.2	47.8
Molprobity clash score, all atoms	1.3 (99^{th} percentile)	1.9 (99^{th} percentile)	0.7 (100^{th} percentile)	0.5 (100^{th} percentile)
Ramachandran plot				
Favored regions (%)	1025 (99.4)	1029 (98.9)	1035 (99.3)	1023 (98.6)
Outlier regions (%)	0	0	0	0
rmsd[c] bond lengths (Å)	0.010	0.005	0.010	0.010
rmsd[c] bond angles (°)	1.210	0.837	1.190	1.150
PDB code	5O4H	5O4J	5O4M	5O4N

[a] Values relative to the highest resolution shell are within parentheses. [b] R_{free} was calculated as the R_{work} for 5% of the reflections that were not included in the refinement. [c] rmsd, root mean square deviation.

References for Supplementary information

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[3] M. D. Winn, C. C. Ballard, K. D. Cowtan, E. J. Dodson, P. Emsley, P. R. Evans, R. M. Keegan, E. B. Krissinel, A. G. W. Leslie, A. McCoy, S. J. McNicholas, G. N. Murshudov, N. S. Pannu, E. A. Potterton, H. R. Powell, R. J. Read, A. Vagin, K. S. Wilson, *Acta Crystallogr. D* **2011**, *67*, 235-242.

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[5] P. Emsley, K. Cowtan, *Acta Crystallogr. D* **2004**, *60*, 2126-2132.

[6] V. B. Chen, W. B. Arendall, J. J. Headd, D. A. Keedy, R. M. Immormino, G. J. Kapral, L. W. Murray, J. S. Richardson, D. C. Richardson, *Acta Crystallogr D* **2010**, *66*, 12-21.